3-Hydroxy Steroid Dehydrogenase Activities of Cortisone Reductase

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The behaviour of various C₁₉ and C₁₈ steroids as substrates for crystalline preparations of cortisone reductase (EC 1.1.1.53) is described. 3 α (Axial,3*R*)-, 3 α (equatorial,3*R*)- and 3 β (axial,3*S*)-hydroxy steroid–NAD oxidoreductase activities are demonstrated. Four pairs of the substrates differed only in the shape of the A/B ring junction, three pairs differed only in substitution at C-10, and four pairs differed only in substitution in ring D. The shape of the substrate molecule and certain substituents (e.g. 10 β -methyl, 17 β -hydroxy, 16-oxo or 17-oxo) altered substrate behaviour, but steroids differing considerably in shape nevertheless acted as substrates, suggesting the possibility of a large or flexible binding site. K_m values varied about 10-fold, many being approx. 140 μ M. V_{max} values covered a greater range (about 200-fold) and the good substrates had high V_{max} values rather than low K_m values.

Hübener *et al.* (1959) described cortisone reductase (EC 1.1.1.53) as a 20 β -hydroxy steroid–NAD oxidoreductase, and in 1965 crystalline preparations of this enzyme were found also to have some 3 α -hydroxy steroid–NAD oxidoreductase activity (J. Jeffery, unpublished work). Kinetic evidence was obtained that the 20 β -hydroxy and 3-hydroxy steroid–NAD oxidoreductase activities were not independent (Gibb & Jeffery, 1971; cf. Gibb & Jeffery, 1972*a*). The 3-hydroxy steroid–NAD oxidoreductase activity appeared not to be limited to 3 α -, 3-axial or 3*R*-hydroxy steroid–NAD oxidoreductase activity (Gibb & Jeffery, 1972*b,c*) and the symmetrical non-steroid adamantanone (which can be regarded as a steroid analogue) acted as a substrate (Gibb & Jeffery, 1972*a*). Reaction rates differed considerably for different substrates (Gibb & Jeffery, 1972*d,e*). Detailed examinations of the reaction products, and estimations of the kinetic constants for a series of steroids differing in the region of the A/B ring junction and in ring D structure, are now described. The nature of the steroid-binding site of the enzyme is considered in the light of the substrate–structure–activity relationships.

Experimental

Materials

Steroids were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), Steraloids Ltd. (Croydon, U.K.), Ikapharm Ltd. (Ramat-Gan, Israel), Imperial Chemical Industries Ltd.

(Macclesfield, Cheshire, U.K.) and the Medical Research Council Reference Collection (Westfield College, London NW3 7ST, U.K.). They were purified by preparative t.l.c. and by crystallization if analytical t.l.c. or m.p. determination (Kofler block) showed purification to be necessary. Crystalline enzyme preparations and NADH were obtained from Fa. Boehringer Mannheim (Mannheim, Germany). General laboratory chemicals and solvents were the best grades available from British Drug Houses Ltd. (Poole, Dorset, U.K.) or Koch–Light Laboratories Ltd. Adsorbents for t.l.c. were from E. Merck A.G. (Darmstadt, Germany).

Enzymic reactions

Enzyme solution. This was made up by diluting a stock suspension (5mg of enzyme/ml of 2.2M-(NH₄)₂SO₄ soln., pH7.0) with 5mM-Tris containing disodium EDTA (1mg/ml), pH8.2.

Coenzyme solution. This was made up by dissolving NADH or NAD⁺ in 10mM-Tris, pH8.2.

Sodium phosphate buffers. These were made by mixing 135mM-Na₂HPO₄ containing disodium EDTA (1.35mM) and 135mM-NaH₂PO₄ containing disodium EDTA (1.35mM) to obtain the required pH.

Incubations for kinetic experiments. Reductions were done at 25°C in cells of 1cm light-path in a model SP.800 double-beam spectrophotometer (Pye–Unicam Instruments, Cambridge, U.K.). The reaction cell contained sodium phosphate buffer, pH7.0 (2ml), 4.05mM-NADH (0.1ml), enzyme solution (volume depending on the amount of enzyme required) and water (to make 0.56ml together with the enzyme solution), dimethyl sulphoxide (0.02ml) and

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steroid substrate in dimethyl sulphoxide (0.02ml). The reference cell contained sodium phosphate buffer, pH 7.0 (2ml), 3.24mM-NADH (0.1 ml), water (0.56ml) and dimethyl sulphoxide (0.04ml). The absorbance at 340nm was recorded continuously, a pen displacement of 1 cm vertically corresponding to reaction of 4.34nmol of NADH and 1 cm horizontally to 8.57s. The amount of enzyme was chosen so that for most substrates 20% of the steroid reacted in 2–4 min. The initial rate was determined by fitting (by eye) a straight line to the part of the recorder trace corresponding to the first 20% of the reaction. The traces were approximately linear along this portion, apart from the initial mixing period.

Oxidations were similarly carried out, but with NAD⁺ (final concn. 1 mM) instead of NADH (final concn. 150 μ M) as above. For 5 α -androstane-3 β ,17 β -diol, 3 β -hydroxy-5 α -androstane-16-one and 3 β -hydroxy-5 α -androstane-17-one as test substrates, sufficient enzyme was used for reaction at 0.2, 0.1 and 0.2% respectively of the rate of the corresponding 3 α -epimers to have been easily detected.

Incubations for preparation of reaction products were carried out in a similar way to that described for kinetic experiments but by using proportionally larger volumes (up to 2.7 litres), in suitable vessels, and using portions of the reaction mixture to follow

the course of reaction spectrophotometrically at 340nm. The conditions used in each case are summarized in Table 1.

Extraction of the enzymic reaction products

The reaction products were extracted with diethyl ether, examined by t.l.c., and either transferred to tubes for micro-scale derivative formation (see below) or purified by preparative t.l.c. With the larger-scale preparations, the purified product was then crystallized from methanol–water.

Characterization of enzymic reaction products

Spectra. These were recorded with the following instruments: i.r., model 237 (Perkin–Elmer Ltd., Beaconsfield, Bucks., U.K.); n.m.r., model HA-100 (Varian Corp., Palo Alto, Calif., U.S.A.); u.v., model SP.800 (Pye–Unicam).

Thin-layer chromatography. T.l.c. plates were coated with an aqueous slurry of silica gel HF₂₅₄₊₃₆₆ or aluminium oxide G to a nominal thickness of 250 μ m (analytical) or 900 μ m (preparative), and were dried at 23°C and activated at 80°C for about 30 min. The chromatograms were developed in closed tanks at 23°C, and the solvent front was allowed to ascend

Table 1. Incubation conditions and extent of reaction for preparation of products with cortisone reductase

Substrate (μ M)	Cofactor (μ M)	Enzyme (μ g/ml)	pH	Dimethyl sulphoxide (%, v/v)	Total volume (ml)	Reaction (%)*
5 α -Androstane-3,16-dione (64)	NADH (455)	0.46	7.0	9.2	542.4	85
17 β -Hydroxy-5 α -oestrane-3-one (100)	NADH (150)	0.15	7.0	1.48	10.8	85
5 α -Oestrane-3,17-dione (50)	NADH (150)	0.15	7.0	1.48	10.8	90
17 β -Hydroxy-5 β -oestrane-3-one (100)	NADH (150)	0.18	7.0	1.48	10.8	85
17 β -Hydroxy-5 α -androstane-3-one (75)	NADH (150)	1.8	7.0	20	2700	85
5 α -Androstane-3,17-dione (64)	NADH (455)	2.8	7.0	9.2	542.4	85
5 α ,10 α -Oestrane-3,17-dione (50)	NADH (150)	3.8	7.0	1.48	10.8	80
17 β -Hydroxy-5 β -androstane-3-one (50)	NADH (150)	9.2	7.0	10	250	90
5 β -Androstane-3,17-dione (100)	NADH (150)	92	7.0	20	250	80
5 β -Androstane-3 α ,17 β -diol (25)	NAD ⁺ (1 mM)	9.2	8.0	10	500	85
5 β -Androstane-3 β ,17 β -diol (25)	NAD ⁺ (1 mM)	23	8.0	10	500	85

* Disappearance of NADH corresponding to complete reduction of one oxo group equals 100% reaction.

to about 15cm above the point of application of the specimen. Aluminium oxide chromatograms were stained by exposing them to HCl vapour for about 10min at 23°C and then either spraying with 2,4-dinitrophenylhydrazine [saturated solution in ethanol–conc. HCl (100:1, v/v)] or spraying with dodecamolybdophosphoric acid–ethanol (1:10, w/v) and heating at about 80°C for 20min. Silica-gel chromatograms were stained with 2,4-dinitrophenylhydrazine as above, but were not exposed to HCl vapour before staining with dodecamolybdophosphoric acid.

Formation of micro-scale derivative

Reduction. The steroid (100–150µg) was dissolved in ethanol (1ml), NaBH₄ (2mg) was added and the mixture stirred for 24h at 23°C. The solution was then centrifuged. To the supernatant one drop of acetic acid was added and solvent was removed in a stream of N₂.

Oxidation. Chromic acid reagent consisted of chromium trioxide (1.8g), H₂SO₄ (2.4ml) and water (to make 10ml). To the steroid (100–150µg) in acetone (0.5ml) was added one drop of chromic acid reagent. The mixture was shaken and then left for 5min at 23°C. Water (5ml) was then added and the mixture extracted with chloroform (10ml). The chloroform extract was washed with 0.1M-NaOH (2ml) and then with water (2ml portions) until the washings were neutral, the extract was dried over Na₂SO₄ and the chloroform removed by rotary evaporation.

Acetylation. The steroid (100–150µg) was dissolved in benzene (0.5ml), acetyl chloride (0.5ml) was added and the mixture left for 1h at 23°C. *n*-Hexane (6ml) was then added, the solution washed with water until neutral, dried over Na₂SO₄, and solvent removed by rotary evaporation.

The application of these methods is exemplified (Table 3) for the substrate 5β-androstane-3,17-dione. The enzymic reaction product (ERP) contained a little residual substrate (plates 1 and 2, *R_F* 0.69 and 0.70) together with materials of lower *R_F* value (plates 1 and 2, *R_F* 0.41 and 0.42; *R_F* 0.58 and 0.59). These were ketones (plate 1) and migrated like 3α-hydroxy-5β-androstan-17-one and 3β-hydroxy-5β-androstan-17-one (plates 1 and 2). They were separated by preparative t.l.c. and the eluted materials were designated ERP-1 and ERP-2 respectively. Neither of these materials was 17α-hydroxy-5β-androstan-3-one or 17β-hydroxy-5β-androstan-3-one (plates 4 and 5, and 7 and 8). The acetylation products of ERP-1 and ERP-2 corresponded to the acetylation products of 3α-hydroxy-5β-androstan-17-one and 3β-hydroxy-5β-androstan-17-one respectively (plates 9–12). Borohydride reduction of ERP-1 and ERP-2 gave material that behaved like 5β-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol respectively (plates 13 and 14), and the acetylation products also corre-

sponded (plates 15–18). The oxidation product of ERP-1 and of ERP-2 behaved like 5β-androstane-3,17-dione (plates 19 and 20). Thus, of the compounds which might be thought to be likely enzymic reaction products from 5β-androstane-3,17-dione and NADH, all were eliminated except 3α-hydroxy-5β-androstan-17-one for ERP-1, and 3β-hydroxy-5β-androstan-17-one for ERP-2. A fortuitous resemblance, which withstood reduction, acetylation, reduction followed by acetylation, and oxidation, is extremely unlikely. The identification of ERP-1 as 3α-hydroxy-5β-androstan-17-one and ERP-2 as 3β-hydroxy-5β-androstan-17-one is therefore regarded as satisfactory. The other identifications by t.l.c. were carried out similarly.

Results

Nature of the reaction

In the presence of NADH, the enzymic reaction with 17β-hydroxy-5α-androstan-3-one gave a product unequivocally identified by t.l.c., m.p., mixed m.p., n.m.r. and i.r. spectra as 5α-androstane-3α,17β-diol. The enzymic reaction products similarly obtained from 5α-androstane-3,16-dione and 5α-androstane-3,17-dione were unequivocally identified by t.l.c., m.p., mixed m.p. and i.r. spectra as 3α-hydroxy-5α-androstan-16-one and 3α-hydroxy-5α-androstan-17-one respectively. No other products were detected. In these cases therefore the course of reaction was the reduction of the 3-oxo group to give the corresponding 3α(axial,3*R*)-hydroxyl group. Consistent with this, no reaction of 5α-androstane-3β,17β-diol, 3β-hydroxy-5α-androstan-16-one or 3β-hydroxy-5α-androstan-17-one was detected in the presence of NAD⁺ and under conditions that would have allowed detection of reaction at 0.2% (or even less) of the rate of reaction of the 3α-epimers.

With the other androstane substrates, the products were examined by t.l.c. of the products themselves and also of derivatives formed from them by micro-scale reduction, oxidation and acetylation, the appropriate authentic compounds being used for comparison in every case. The enzymic reaction with 17β-hydroxy-5β-androstan-3-one in the presence of NADH gave a product shown in this way to be indistinguishable from 5β-androstane-3α,17β-diol. The corresponding 3β,17β-diol was not detected. Nevertheless, both 5β-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol were substrates in the presence of NAD⁺ and the reaction product in each case was not distinguished from 17β-hydroxy-5β-androstan-3-one. The enzymic reaction with 5β-androstane-3,17-dione in the presence of NADH gave two products which t.l.c. examination showed to be 3α-hydroxy-5β-androstan-17-one and 3β-hydroxy-5β-androstan-17-one. The course of reaction in these cases therefore included both 3α(equatorial,3*R*) and 3β(axial,3*S*) activities.

Table 2. *Kinetic constants for the reduction of various 3-oxo steroids by cortisone reductase*
 Conditions: 150 μM -NADH, 25°C, pH 7.0, 1.48% dimethyl sulphoxide.

Reference	Substrate (μM)	Correlation coefficient (1/v upon 1/[S])	Apparent K_m (μM)		Apparent V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)		K_m/V_{max}	
			Non-weighted	Weighted $\pm 2 \times \text{s.e.m.}$	Non-weighted	Weighted $\pm 2 \times \text{s.e.m.}$	Non-weighted	Weighted $2 \times \text{s.e.m. range}$
<i>a</i>	5 α -Androstane-3,16-dione (30-100)	0.96	85	90 $\pm$ 26	180	188 $\pm$ 32	0.47	0.48 0.29-0.74
<i>b</i>	17 β -Hydroxy-5 α -oestrane-3-one (30-100)	0.98	143	126 $\pm$ 40	147	134 $\pm$ 27	0.97	0.94 0.53-1.55
<i>c</i>	5 α -Oestrane-3,17-dione (30-100)	0.99	425	599 $\pm$ 245	134	184 $\pm$ 68	3.2	3.2 1.4-7.3
<i>d</i>	17 β -Hydroxy-5 β -oestrane-3-one (30-100)	0.99	472	844 $\pm$ 424	86	147 $\pm$ 70	5.5	5.7 2.0-16
<i>e</i>	17 β -Hydroxy-5 α -androstan-3-one (25-75)	0.94	162	112 $\pm$ 38	18	14 $\pm$ 3.4	8.8	8.0 4.2-14
<i>f</i>	5 α -Androstane-3,17-dione (30-100)	0.97	150	130 $\pm$ 29	3.0	2.7 $\pm$ 0.41	50	48 32-69
<i>g</i>	5 α ,10 α -Oestrane-3,17-dione (30-100)	0.99	134	136 $\pm$ 17	1.3	1.3 $\pm$ 0.11	103	103 84-128
<i>h</i>	17 β -Hydroxy-5 β -androstan-3-one (30-100)	0.97	184	224 $\pm$ 86	0.84	0.98 $\pm$ 0.29	219	229 109-449
<i>i</i>	5 β -Androstane-3,17-dione	—	—	—	—	—	High	High

The reaction products from the oestrane compounds were not identified because of insufficient material and lack of authentic reference specimens. However, the enzymic reaction product from 17 β -hydroxy-5 β -oestrane-3-one behaved on t.l.c. like the chemical reduction product prepared by micro-scale NaBH₄ reduction. The course of this chemical reduction is known (Kirk & Hartshorn, 1968) and the expected product is 5 β -oestrane-3 α ,17 β -diol. The enzymic reaction product from 17 β -hydroxy-5 α -oestrane-3-one was distinguished from the chemical reduction product prepared by micro-scale complete reduction of 5 α -oestrane-3,17-dione, expected to be 5 α -oestrane-3 β ,17 β -diol (Kirk & Harshorn, 1968). Thus for 17 β -hydroxy-5 α -oestrane-3-one and 17 β -hydroxy-5 β -oestrane-3-one there was no reason to suppose that the enzymic reaction did not follow the same course as for the corresponding androstane compounds, giving the 3 α -hydroxy compounds. The enzymic reaction with 5 α -oestrane-3,17-dione gave material which appeared as a single spot on t.l.c., stained with 2,4-dinitrophenylhydrazine and was probably a hydroxyoestrane, though there was no evidence as to whether it was 3 α -hydroxy-5 α -oestrane-17-one. 5 α ,10 α -Oestrane-3,17-dione gave more than one product.

Rate of reaction

Care was taken that no precipitation of steroid occurred during the kinetic experiments. (Precipitation corresponding to a 5 μ M fall in steroid concentration was easily detectable under the conditions used.) 17 β -Hydroxy-5 α -androstane-3-one was seen to come out of solution above 75 μ M, so measurements were limited to 25–75 μ M for this substrate. For other steroids, the range 30–100 μ M was used. Initial rates of reaction (v) were determined in duplicate at each of six substrate concentrations, and every such experiment was repeated to give 24 'points' for each substrate. In each case, the reciprocal of v was linearly related to the reciprocal of the substrate concentration (correlation coefficients, Table 2). The apparent K_m (Michaelis constant) and apparent V_{max} (maximum velocity) were calculated by non-weighted regression analysis (cf. the graphical method of Lineweaver & Burk, 1934) and by the weighting procedure of Wilkinson (1961). The ratio K_m/V_{max} serves as a parameter of efficiency of utilization of the substrate, and in Table 3 the substrates are arranged in order of increasing K_m/V_{max} (i.e. decreasing efficiency of utilization). The maximum $2 \times \text{s.e.m.}$ range of K_m/V_{max} was calculated by using $(K_m - 2 \times \text{s.e.m.})/(V_{max} + 2 \times \text{s.e.m.})$ and $(K_m + 2 \times \text{s.e.m.})/(V_{max} - 2 \times \text{s.e.m.})$. The order of increasing K_m/V_{max} (Table 3) was not uniformly the order of increasing K_m but was roughly the order of decreasing V_{max} . For eight substrates (a–h), K_m/V_{max} varied about

500-fold, K_m about 10-fold and V_{max} about 200-fold. 5 β -Androstane-3,17-dione was an extremely poor substrate (initial rate 0.0044 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 50 μ M) and kinetic constants were not obtained. No inhibition by this compound of the reaction of 17 β -hydroxyoestr-5(10)-en-3-one (10 μ M) was detected under conditions that would have allowed detection of simple competitive inhibition with K_i less than 550 μ M. It seems likely therefore that 5 β -androstane-3,17-dione had a high K_m/V_{max} not attributable solely to a low V_{max} .

Discussion

Four pairs of compounds (Table 2, compounds *b* and *d*, *e* and *h*, *f* and *i*, and *c* and *g*) differed only in the shape of the A/B ring junction. The A/B *trans* compound was the better substrate of each pair, though this was attributable variously to its lower K_m (*b* and *d*), its higher V_{max} (*c* and *g*), or both (*e* and *h*, *f* and *i*). Compounds *g* and *i* are A/B *cis* in different ways, and the relation of the 3-oxo group to the rest of the molecule is different in these two compounds, and also differs from the 5 α ,10 β compounds.

The only structural difference between compounds *b* and *e*, *c* and *f*, and *d* and *h* was the presence or absence of the 10 β -methyl group. The 10 β -methyl compound was the less efficiently utilized of each pair because of its much lower V_{max} value, though this effect was mollified in two cases (*f* and *h*) by lower K_m values.

The change from a 17 β -hydroxy to a 17-oxo group was the only structural difference for the pairs of compounds *b* and *c*, *e* and *f*, and *h* and *i*. In each case the 17-oxo compound was utilized less efficiently. Two of these pairs (*b* and *c*, and *e* and *f*) are of the same structural type (5 α ,10 β , all-chair, all-*trans*) and it is noteworthy that for one pair (*b* and *c*) the V_{max} values were similar and the K_m values different, whereas for the other pair (*e* and *f*) the reverse was the case.

Consideration of the shapes and structures of the compounds studied indicates that the enzyme does not provide a binding site like a template on which, in every case, an extended region of the substrate is uniquely bound. Reaction of the various substrates is undoubtedly closely related, and probably the simplest explanation (though not the only possible explanation) would be that the same active centre is involved. If this is the case, it must permit binding in various ways. The non-steroidal but analogous compound adamantanone was a poorer substrate (K_m/V_{max} about 1300) (Gibb & Jeffery, 1972a) than the steroids described here (except, perhaps, compound *i*), so that, even among these poor substrates, parts of the molecule other than the A ring evidently facilitate utilization as a substrate. These facts suggest that the binding region comprises a hydrophobic pocket. However, the marked difference of behaviour of com-

Table 3. *An example of the characterization of enzymic reaction products by t.l.c.*

5 β -Androstane-3,17-dione reacted with cortisone reductase and excess of NADH (see Table 1 and the Experimental section). The enzyme reaction products (ERP) and derivatives formed from them on a micro-scale were chromatographed on silica gel (S) or aluminium oxide (A) in the following systems: (1) hexane-ethyl acetate (1:1, v/v); (2) hexane-ethyl acetate (2:3, v/v); (3) hexane-ethyl acetate (5:1, v/v); (4) hexane-ethyl acetate (2:1, v/v); (5) chloroform-dichloromethane-ethanol (49:50:1, by vol.). The plates were stained with 2,4-dinitro-phenylhydrazine reagent (K) or dodecamolybdophosphoric acid reagent (H).

Specimen	No. of spots observed	R_F	Chromatographic system
ERP	3	0.41 0.58 0.69	S(1)K Plate 1
3 β -Hydroxy-5 β -androstan-17-one	1	0.57	
3 α -Hydroxy-5 β -androstan-17-one	1	0.41	
5 β -Androstane-3,17-dione	1	0.70	
ERP	3	0.42 0.59 0.70	S(1)H Plate 2
3 β -Hydroxy-5 β -androstan-17-one	1	0.60	
3 α -Hydroxy-5 β -androstan-17-one	1	0.42	
5 β -Androstane-3,17-dione	1	0.70	
ERP-1	1	0.39	S(1)H Plate 3
3 β -Hydroxy-5 β -androstan-17-one	1	0.57	
3 α -Hydroxy-5 β -androstan-17-one	1	0.39	
ERP-1 + 3 α -hydroxy-5 β -androstan-17-one	1	0.39	
ERP-1	1	0.38	S(1)H Plate 4
17 α -Hydroxy-5 β -androstan-3-one	1	0.54	
ERP-1 + 17 α -hydroxy-5 β -androstan-3-one	2	0.37 0.54	
ERP-1	1	0.36	S(1)H Plate 5
17 β -Hydroxy-5 β -androstan-3-one	1	0.52	
ERP-1 + 17 β -hydroxy-5 β -androstan-3-one	2	0.37 0.54	
ERP-2	1	0.61	S(1)H Plate 6
3 β -Hydroxy-5 β -androstan-17-one	1	0.60	
3 α -Hydroxy-5 β -androstan-17-one	1	0.42	
ERP-2 + 3 β -hydroxy-5 β -androstan-17-one	1	0.61	
ERP-2	1	0.58	S(1)H Plate 7
17 α -Hydroxy-5 β -androstan-3-one	1	0.53	
ERP-2 + 17 α -hydroxy-5 β -androstan-3-one	2	0.52 0.58	
ERP-2	1	0.58	S(1)H Plate 8
17 β -Hydroxy-5 β -androstan-3-one	1	0.52	
ERP-2 + 17 β -hydroxy-5 β -androstan-3-one	2	0.51 0.59	
Acetylated ERP-1	1	0.24	S(3)H Plate 9
Acetylated 3 β -hydroxy-5 β -androstan-17-one	1	0.19	
Acetylated 3 α -hydroxy-5 β -androstan-17-one	1	0.23	
Acetylated ERP-2	1	0.19	S(3)H Plate 10
Acetylated 3 β -hydroxy-5 β -androstan-17-one	1	0.20	
Acetylated 3 α -hydroxy-5 β -androstan-17-one	1	0.25	

Table 3.—*Continued*

Specimen	No. of spots observed	R_F	Chromatographic system
Acetylated ERP-1	1	0.31	S(5)H
Acetylated 3 β -hydroxy-5 β -androstane-17-one	1	0.17	Plate 11
Acetylated 3 α -hydroxy-5 α -androstane-17-one	1	0.30	
Acetylated ERP-2	1	0.19	S(5)H
Acetylated 3 β -hydroxy-5 β -androstane-17-one	1	0.18	Plate 12
Acetylated 3 α -hydroxy-5 β -androstane-17-one	1	0.31	
NaBH ₄ -reduced ERP-1	1	0.41	S(2)H
5 β -Androstane-3 β ,17 β -diol	1	0.56	Plate 13
5 β -Androstane-3 α ,17 β -diol	1	0.40	
NaBH ₄ -reduced ERP-1 + 5 β -androstane-3 α ,17 β -diol	1	0.41	
NaBH ₄ -reduced ERP-2	1	0.58	S(2)H
5 β -Androstane-3 β ,17 β -diol	1	0.58	Plate 14
5 β -Androstane-3 α ,17 β -diol	1	0.43	
NaBH ₄ -reduced ERP-2 + 5 β -androstane-3 β ,17 β -diol	1	0.59	
Acetylated NaBH ₄ -reduced ERP-1	1	0.31	S(3)H
Acetylated 5 β -androstane-3 β ,17 β -diol	1	0.27	Plate 15
Acetylated 5 β -androstane-3 α ,17 β -diol	1	0.31	
Acetylated NaBH ₄ -reduced ERP-2	1	0.27	S(3)H
Acetylated 5 β -androstane-3 β ,17 β -diol	1	0.28	Plate 16
Acetylated 5 β -androstane-3 α ,17 β -diol	1	0.33	
Acetylated NaBH ₄ -reduced ERP-1	1	0.46	S(4)H
Acetylated 5 β -androstane-3 β ,17 β -diol	1	0.35	Plate 17
Acetylated 5 β -androstane-3 α ,17 β -diol	1	0.46	
Acetylated NaBH ₄ -reduced ERP-2	1	0.32	S(4)H
Acetylated 5 β -androstane-3 β ,17 β -diol	1	0.33	Plate 18
Acetylated 5 β -androstane-3 α ,17 β -diol	1	0.45	
Oxidized ERP-1	1	0.68	A(4)K
5 β -Androstane-3,17-dione	1	0.68	Plate 19
5 α -Androstane-3,17-dione	1	0.77	
Oxidized ERP-2	1	0.68	A(4)K
5 β -Androstane-3,17-dione	1	0.68	Plate 20
5 α -Androstane-3,17-dione	1	0.77	

pounds *a* and *f*, caused by the relatively slight structural difference of a 16-oxo instead of a 17-oxo group, suggests a specific interaction with a polar group in this case. Polar interactions in this region may, for example, slightly alter the alignment of the steroid on the binding site. This could explain the change mainly in the apparent V_{max} and relatively slight change in apparent K_m , for compounds *a* and *f*, and *e* and *f*. On the other hand, hydrophobic interactions involving the β side of the molecule in the region around C-10 may also modify alignment of the 3-oxo group at the catalytic site (compare, for example, compounds *b* and *e*, *c* and *f*, and *d* and *h*). Consistent with this, an adverse effect of a 1 β -methyl group was previously observed (Gibb & Jeffery, 1972*d*). Overall, it is striking that the

good substrates had high V_{max} values rather than low K_m values, suggesting that what distinguished them was probably not the extent of their binding, but the precise way in which they bound. Comparison could be made with the concept of drugs having similar affinities for their receptor, but different 'intrinsic activity' (Ariëns, 1954).

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